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PROLINE BETAINE: A UNIQUE OSMOLYTE IN AN EXTREMELY EURYHALINE OSMOCONFORMER

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Abstract

The extremely euryhaline mollusc, *Elysia chlorotica,* does not utilize intracellular free amino acids for cell volume regulation during osmotic stress. Instead, *Elysia* utilizes an osmolyte previously unknown from animals, proline betaine. Although proline betaine occurs in some plants and *Elysia* forms a symbiosis with an algae, the proline betaine in *Elysia* seems to be a product of the animal.

Introduction

Two important aspects of the mechanisms utilized by cells to control intracellular water and, thereby, to survive an osmotic stress have been discovered during the past few years. First, there is mounting evidence that the cells of marine animals, traditionally held to use small molecular weight organic osmolytes such as free amino acids, have additional inorganic ionic components to the solute movements which regulate cellular volume following an osmotic stress (Pierce, 1982; Warren and Pierce, 1982; Costa and Pierce, 1983). Second, the cells of some extremely salinity tolerant invertebrate species use intracellular organic osmolytes which are quite different from the free amino acids usually encountered in less euryhaline species. For example, the cells in *Limulus polyphemus* utilize the quaternary ammonium compound glycine betaine as intracellular solute to sustain cell volumes in salinities ranging from 5% to 200% sea water (Warren and Pierce, 1982). For another example, the ascoglossan opisthobranch mollusc, *Elysia chlorotica* is the most euryhaline osmoconformer known (Pierce *et al.,* 1983b). It will survive from salinities approaching freshwater (24 mosm) up to 250% SW (2422 mosm) and is an excellent volume regulator. The intracellular amino acid pool size in *Elysia* is small, even smaller than that found in *Limulus* (Pierce *et al.,* 1983b). Thus, instead of amino acids *Elysia* utilizes a different osmolyte, but it is a molecule of unknown structure. Since the euryhalinity of *Elysia* suggests both a potential value as a model cell volume regulatory system and the possibility that the molecule may impart the extreme cellular osmotic tolerance, we have purified it from *Elysia* tissues and identified it.

Materials and Methods

Purification

Preliminary measurements indicated that the *Elysia* osmolyte could be precipitated by acid reineckate and gave a positive response to Dragendorff's reagent. The acid reineckate precipitation technique used was modified from that of Barnes and Black-

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stock (1974) and is described in detail elsewhere (Warren and Pierce, 1982). The Dragendorff's test was performed using the EtOH-NH_4OH thin layer chromatography protocol described below which is modified from Bregoff *et al.* (1953). Although not conclusive proof, these two characteristics indicated that the unknown molecule was a quaternary ammonium compound. With that as a hypothesis, we were able to purify a Dragendorff's positive substance from *Elysia* tissues using the following protocol. First, several animals were frozen on dry-ice, freeze dried, and weighed. Then, 0.5 to 1.5 gm of dry tissue were homogenized in 40% EtOH. The homogenate was brought to a boil in a water bath and then centrifuged at 20,000 $\times$ *g* for 20 min. The pellet was discarded and the supernatant shaken with ether in a separatory funnel. The ether and aqueous phases were allowed to separate and the aqueous phase collected and freeze dried. The residue was dissolved in distilled water and applied to a mixed bed ion-exchange column of Dowex-1 and Amberlite-50 (2:1). The column was eluted with five volumes of distilled water and the eluate collected as a single fraction. The fraction was freeze dried, dissolved in a small amount of 70% EtOH, and streaked across the bottom of a cellulose TLC plate. The plate was developed in EtOH-NH_4OH (8.5:1.5), dried, and the edges sprayed with Dragendorff's reagent. The TLC plate was scraped between the Dragendorff positive spots and the removed cellulose then extracted with distilled water and centrifuged at 20,000 $\times$ *g* for 20 min. The supernatant was freeze dried, the residue dissolved in EtOH and again streaked on a TLC plate. This plate was developed in BuOH, HAC, H_2O (10:3:7) dried, and sprayed with Dragendorff's reagent as before. Again, the plate was scraped, the removed cellulose extracted with water and then centrifuged at 20,000 $\times$ *g* for 20 min. Finally the supernatant was freeze dried. This purification procedure produced a brown freeze-dried residue which had a consistency of hard caramel and was extremely hygroscopic.

Identification and quantification

The residue from the purification was subjected to a variety of analytical procedures: proton and carbon nuclear magnetic resonances (NMR), UV and IR spectra. The results of these analyses suggested that the substance we purified from *Elysia* was N,N-dimethyl proline perhaps better known as proline betaine or stachydrine. The identification was confirmed by comparing the ^{1}H-NMR spectra, the high performance liquid chromatography (HPLC), and the thin-layer chromatograms of the purified material with synthetic proline betaine which had been taken through our purification protocol. Proline betaine was synthesized by heating proline and methyl iodide according to the method of Pettigrew and Smith (1977). Proline betaine was quantified by an HPLC technique (Altex Model 334 equipped with a Gilson Holochrome variable wavelength detector). The analytical column was 10 cm ODS with 3 μM diameter particles (Microsorb-short one, Rainin Instruments). Proline betaine was separated using 0.0125 *M* NaH_2PO_3 (pH 4.95) as the mobile phase and was detected at 200 n*M*. We also had success with a C_6 20 cm column (Spherisorb, Chromanetics, Inc.), but the analytical time is longer with this longer column.

Using HPLC, we have measured the amount of proline betaine in whole animal homogenates of *Elysia* adapted to salinities ranging from 87 mosm to 1834 mosm for two weeks.

Results and Discussion

The NMR spectra of the native material (Fig. 1) suggested that the *Elysia* osmolyte was proline betaine and, indeed, in all of our tests including H-NMR the native

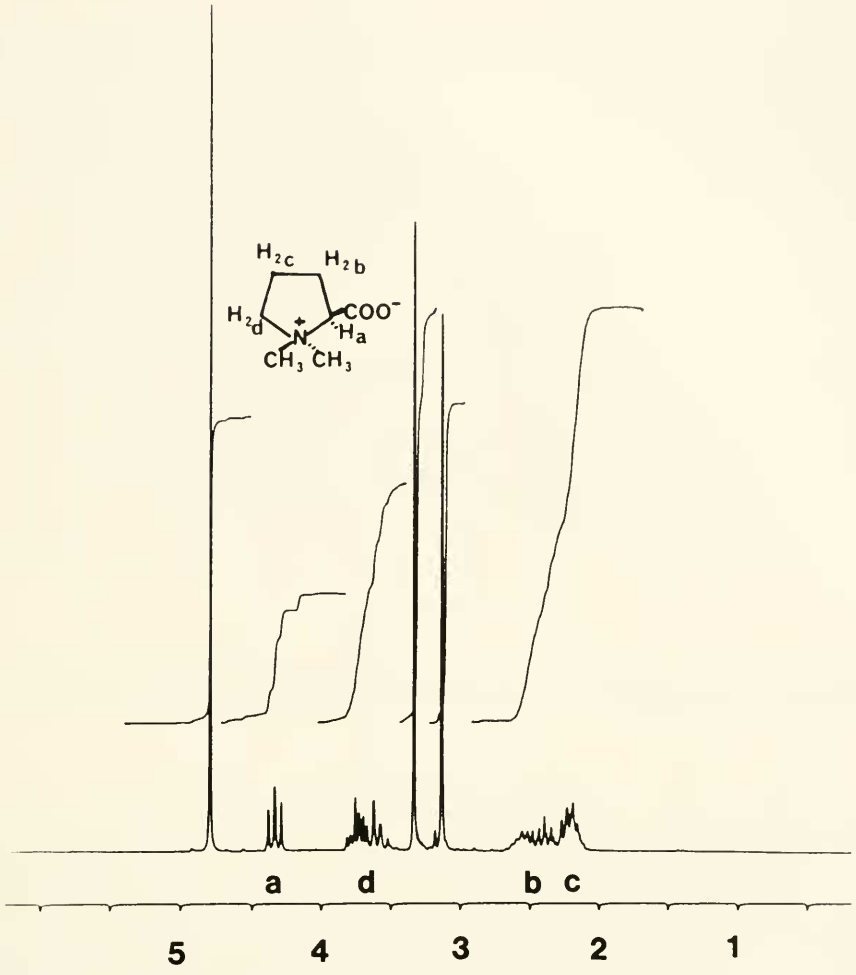

FIGURE 1. NMR spectrum of the native proline betaine purified from *Elysia chlorotica*. The letters under the peaks indicate the position in the spectrum of the correspondingly labeled protons in the structural diagram. The numbers indicate chemical shifts in ppm with respect to the external standard, sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). The proline betaine was dissolved in D_2O and the peak at 4.8 corresponds to HOD. Spectra were recorded at 22° on an IBM WP 200 MHz NMR spectrometer.

material was identical to the authentic standard. Therefore, the molecule we extracted and purified from *Elysia* is proline betaine, contrary to an earlier report (Pierce *et al.*, 1983a).

Proline betaine concentrations were 613 μmole/g dry wt in the tissues of *Elysia* adapted to full strength sea water (926 mosm). Furthermore, the concentration of proline betaine varied with salinity ranging from 190 μmoles/g dry wt in 87 mosm to 1041 μmoles/g dry wt in 1834 mosm, the lowest and highest salinities tested (Fig. 2). Therefore, the concentration of proline betaine is of an appropriate magnitude to be the major intracellular osmolyte and its behavior with salinity adaptation indicates utilization of proline betaine for cell volume regulation. Indeed, we have shown elsewhere (Rowland and Pierce, 1984) that a pulse of proline betaine appears in the

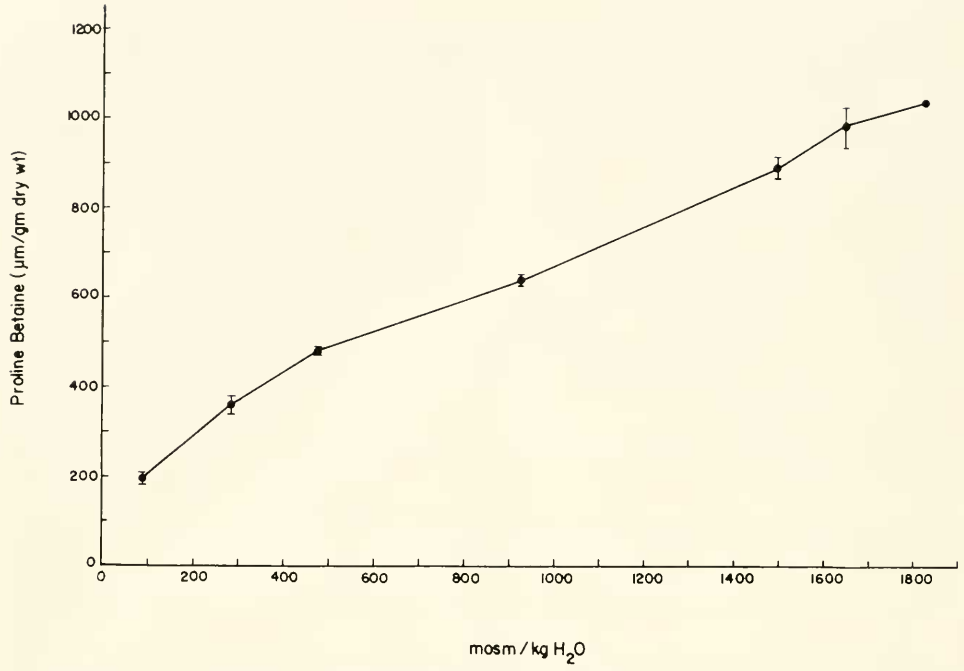

FIGURE 2. Concentrations of proline betaine in *Elysia chlorotica* adapted to various salinities for 14 days. Each point is a mean from five animals. Error bars indicate ±S.E.M.

blood of low salinity stressed *Elysia,* indicating a release of the molecule from intracellular stores in response to the stress.

Proline betaine is virtually unknown from animals, so at present nothing is known with regard to its biochemistry. Trace amounts of proline betaine have been tentatively identified by paper chromatography in the adductor muscle of *Atrina pectinata* (Hayashi and Kondsu, 1977) and by mass spectral analysis in the ovary of *Callista brevisiphonata* (Yasumoto and Shimizu, 1977). Proline betaine has been found occasionally in plants both in algae (Blunden *et al.,* 1982) and higher plants (Cornforth and Henry, 1952; Ahmad *et al.,* 1974; Sethi and Carew, 1974; Ahman and Basha, 1975) from both marine and terrestrial environments. There is almost no information on proline betaine metabolism in plants either, except that alfalfa shoots did not produce proline betaine from radioactively labelled proline (Robertson and Marion, 1959). However, *Elysia chlorotica* forms a symbiosis with the green alga *Vaucheria.* The slug seems to feed only on *Vaucheria* filaments and, as it feeds, incorporates the chloroplasts from the algae into certain cells lining the walls of the digestive tubules. The chloroplasts cause the emerald-green color of *Elysia* and also remain functional for months in the animal tissue (West *et al.,* 1984). Since proline betaine seems to be much more common to plants than animals, the possibility existed that the proline betaine in *Elysia* was arising out of the symbiosis with *Vaucheria.* An algal extract run through our HPLC protocol did not result in a peak which co-eluted with a spike of proline betaine. These results indicate that proline betaine is not present in the algae. Three additional observations also indicate that the proline betaine is produced by *Elysia* itself. First, the animals used in the salinity experiment (Fig. 2) above were not fed

during the experiment and proline betaine still increased in the animals exposed to salinities above control. Second, the *Elysia* brain which lacks chloroplasts still contains the high amounts of proline betaine. Third, *Elysia* which have been reared from eggs in the laboratory and kept in incubators for months and fed only small amounts of *Vaucheria* have the same tissue concentrations of proline betaine as freshly collected field animals (595 ± 46.0 μmoles/g dry wt and 613 ± 9.3 μmoles/g dry wt, respectively). We cannot at present conclusively eliminate the symbiotic chloroplast as the source of proline betaine, but our observations do rule out the plant as a source, and the osmolyte is an unlikely molecule for synthesis by a chloroplast.

Finally, it is unclear at present why *Elysia* should use such a unique compound as proline betaine. There is some botanical evidence that glycine betaine somehow protects some enzymes from salt inhibition (Pollard and Wyn Jones, 1979). Further in their review, Yancey *et al.* (1982) suggested that methylamines are useful osmolytes as a result of their counteraction of the protein perturbation effects of high salts or urea concentrations. *Elysia* does not accumulate urea so that possibility is unlikely. However, the possibility that, in addition to a role as an osmolyte, proline betaine may be useful as a protector of intracellular proteins from the effects of whatever intracellular salt concentrations are reached in 200% sea water seems worth testing in *Elysia*.

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